Brief Definitive Report

T Cell–dependent Induction of NF-κB in B Cells

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Summary

In comparison to B cell stimulation mediated by surface immunoglobulin (Ig) antigen receptor ligation, little is known about the intracellular events associated with T cell–dependent B cell responses. A model for the efferent phase of T cell–B cell interaction was used to examine the capacity of activated T cells to trigger nuclear expression of the *trans*-acting transcription factor, NF-κB, in B cells. Fixed, activated, but not fixed, resting Th2 cells were found to induce increased binding activity for a κB site–containing oligonucleotide in a time-dependent manner. This induction of NF-κB was eliminated by an antibody directed against a 39-kD cell interaction protein on activated T cells as well as by a soluble form of B cell CD40. Of particular relevance to intracellular signaling, NF-κB induction was not diminished by prior depletion of B cell protein kinase C (PKC) with phorbol myristate acetate. These results strongly suggest that T cell–dependent B cell stimulation is associated with NF-κB induction via p39–CD40 interaction and that this is brought about by non-PKC dependent signaling, in marked contrast to the previously documented requirement for PKC in sIg receptor-mediated stimulation. This suggests that NF-κB responds to more than one receptor-mediated intracellular signaling pathway in B cells and may be part of a "final common pathway" for B cell stimulation.

The serological response to monomeric protein antigens depends on the cooperative behavior of both T and B cells, encompassing antigen processing and presentation by B cells, specific recognition of antigen in the context of MHC class II by T cells, and resultant mutual stimulation of both interacting parties (for review see reference 1). The development of model systems for T-dependent B cell responses has provided the means to dissect these events. It has been shown that B cells can be stimulated by T cells in the absence of prior antigen processing, and that T cell stimulation results in the appearance of antigen- and MHC-independent B cell stimulating activity (1). The B cell stimulating activity is recovered in formaldehyde fixed cell preparations as well as in purified membranes obtained from stimulated T cells (1). This activity is associated with the acquisition of a 39-kD protein that interacts with B cell CD40 (1–3). Despite progress in identifying the ligand–receptor interactions associated with T-dependent B cell responses, relatively little is known about the intracellular events triggered by T cells that ultimately induce B cells to exit the resting state and enter cell cycle.

The cognate binding site for the *trans*-acting transcription factor, NF-κB, is found in the promoters of a number of genes of immunological interest, including Igκ, IL-2Rα, and IL-6 (4). In a polyclonal model for T-independent B cell stimulation, we have demonstrated that surface Ig (sIg) receptor ligation mediates nuclear expression of NF-κB in a PKC-dependent and protein synthesis-independent fashion, suggesting that NF-κB may function as a "third messenger" that transduces cytoplasmic protein kinase signals to nuclear transcriptional events (5). In the present study we examined the regulation of NF-κB during T-dependent B cell stimulation in order to discern nuclear events that may be associated with T–B cell interaction.

Materials and Methods

Animals. BALB/cByJ mice were obtained from The Jackson Laboratory (Bar Harbor, ME).

Preparation of Primary B Cells. Spleen cells from 2–4-mo-old naive mice were depleted of T cells and macrophages by treatment with anti-Thy-1.2 antibody plus complement and by plate adherence, respectively, as previously described (6). RBC and nonviable cells were removed by sedimentation over Lympholyte-M (Cedarlane, Ontario, Canada). The resulting B cells were cultured in RPMI 1640 medium (BioWhittaker, Inc., Walkersville, MD) supplemented with 5% FCS (Sigma Chemical Co., St. Louis, MO), 10 mM Hepes (Calbiochem-Novabiochem, San Diego, CA), 5×10^{-5} M 2-ME (Sigma Chemical Co.), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine.

Maintenance of Th2 Cells. The rabbit Ig–specific T cell line CDC35 (I-A^d restricted) was propagated biweekly with antigen

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and irradiated spleen cells (7). CDC35 Th2 cells were used 2–3 wk after their last restimulation and were purified before use by centrifugation over Ficoll-Hypaque (Pharmacia, Uppsala, Sweden).

Activation and Fixation of Th2 Cells. T cells were activated by overnight incubation with plate adherent monoclonal hamster anti-mouse CD3 antibody (145-2C11) and then fixed with 0.5% formaldehyde, as previously described (8).

Proliferation Assay. Splenic B cells (10^5 in 0.2 ml) were cultured in flat bottomed microtiter wells in quadruplicate with an equal number of fixed, activated (or fixed, resting) T cells, or with other reagents, as indicated, for 48 h. Tritium incorporation was assessed after exposure to 0.5 μCi [^{3}H]thymidine (20 Ci/mmol; New England Nuclear, Boston, MA) during the last 6 h of culture, as described (9).

Preparation of Protein Kinase C (PKC)-depleted B Cells. Splenic B cells were treated with PMA at 100 ng/ml for 20 h (10). PMA-treated B cells were washed in complete medium, after which nonviable cells were removed by sedimentation over Lympholyte M. The wash medium and Lympholyte M contained PMA at 100 ng/ml to avoid restoration of PKC.

Preparation of Nuclear Extracts. Splenic B cells were cultured (10^7 in 5 ml) in wells of 6-well flat bottomed plates (Costar Corp., Cambridge, MA) in the presence or absence of an equal number of fixed, activated (or fixed, resting) T cells or with other reagents, as indicated. B cells were lysed in a hypotonic buffer and nuclei extracted in 430 mM NaCl with protease inhibitors, as described (5). Protein concentrations were determined by the Bradford method (11) (Bio-Rad Laboratories, Richmond, CA).

Electrophoretic Mobility Shift Assay (EMSA). Double-stranded oligonucleotides containing the κB sequence (GGGACTTTCC) or a mutated κB sequence (TTCACTTTCC) cloned into the BamHI site of PUC13 and PTZ18R plasmids respectively, were kindly provided by Dr. Ranjan Sen (Brandeis University, Waltham, MA). The oligonucleotide fragments were end-labeled with α-[^{32}P]dATP and binding reactions and nondenaturating gel electrophoresis were carried out as previously described (5). Within a given experiment, equal amounts of nuclear protein were added to each binding reaction.

Reagents. F(ab')$_2$GaMIgM was obtained from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). LPS from *Salmonella typhimurium* and PMA were obtained from Sigma Chemical Co. MR-1 monoclonal hamster anti-mouse p39 antibody was a precious gift from Dr. R. J. Noelle (Dartmouth Medical School, Lebanon, NH). Soluble CD40-Ig fusion protein was obtained from a cell line kindly provided by Dr. Marilyn Kehry (Castle, B. E., K. Kishimoto, M. L. Brown, and M. R. Kehry, manuscript submitted for publication).

Results

Induction of κB-binding Activity in B Cells Stimulated by Fixed, Activated T Cells (T^a). Cells of the CDC35 Th2 cell line were activated by surface-bound anti-CD3 antibody and fixed with formaldehyde as described in Materials and Methods. T^a cells failed to exclude trypan blue. In coculture experiments, T^a stimulated primary B cells to incorporate substantial amounts of [^{3}H]thymidine, while fixed, nonactivated or resting T cells (T^r) did not (data not shown and see Figure 3 *B*).

To determine whether the influence of activated T cells on B cells includes induction of NF-κB, nuclear extracts were obtained and analyzed by EMSA using a κB-containing oligonucleotide, as previously described (5). A detectable (baseline) level of nuclear κB-binding activity was observed in primary B cells cultured with medium alone (Fig. 1). When T^a were added (1:1), expression of nuclear κB-binding activity by B cells increased with time, reaching a plateau at 4–6 h. This maximum occurred later than that observed with sIg receptor ligation (5), and was as great as the induction produced by cross-linking anti-Ig antibody (10 μg/ml) at 2 h. T^r had no effect on B cell κB-binding activity, either at 2 h as shown, or at 4 h (data not shown). Further, the measured κB-binding activity was not extracted from T^a alone (Fig. 1). Thus, κB-binding activity is specifically induced in B cells by T^a.

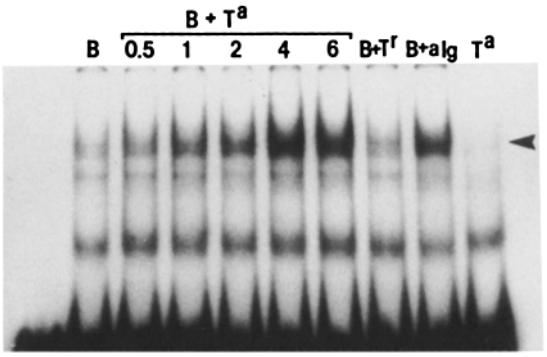

Figure 1. Induction of κB-binding activity in B cells stimulated by fixed, activated T cells. Nuclear extracts were prepared from primary splenic B cells (*B*) alone or from B cells incubated with either, an equal number of fixed, activated T cells (T^a) for the indicated times in hours, an equal number of fixed, nonactivated or resting T cells (T^r) for 2 h, or F(ab')$_2$GaMIgM (*aIg*) at 10 μg/ml for 2 h. Nuclear extracts were also prepared from T^a alone. Nuclear extracts were tested by EMSA, using a κB site-containing oligonucleotide probe, as described in Materials and Methods. The unmarked lane represents κB probe alone. (◄) Position of specific κB-binding activity. One of two comparable experiments is shown.

The κB-binding activity extracted from T cell–stimulated B cells was specifically competed by unlabeled native (wild-type) κB-containing oligonucleotide but not by an oligonucleotide containing a mutated κB site (data not shown). On the basis of the specificity of oligonucleotide binding as well as the electrophoretic mobility of induced nucleoprotein complexes (which parallels that of constitutive and anti-Ig–induced nucleoprotein complexes), we concluded that the T^a-induced κB-binding activity detected by EMSA represents NF-κB or a closely related complex.

Inhibition of NF-κB Induction by Anti-p39 Antibody and CD40-Ig. To determine whether T cell p39 plays a role in the induction of B cell NF-κB, monoclonal hamster anti-p39 antibody was included in the culture with B cells and T^a. This led to inhibition of T^a-induced NF-κB expression as shown in Fig. 2 *A*. At the same time, monoclonal hamster anti-CD3 antibody, used here as a specificity control, had no effect. To evaluate the involvement of interaction with B cell CD40, soluble CD40-Ig fusion protein (Castle, B. E., et al., manuscript submitted for publication) was included in the culture with B cells and T^a. This also led to inhibition of T^a-induced NF-κB expression as shown in Fig. 2 *B*. This inhibition was specific inasmuch as CD40-Ig did not block induc-

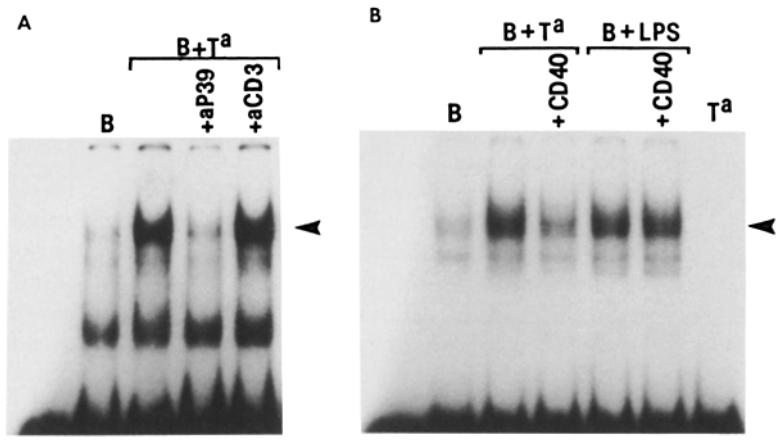

Figure 2. Inhibition of NF-κB induction by anti-p39 antibody and by CD40-Ig. (*A*) Primary splenic B cells (*B*) were incubated with an equal number of fixed, activated T cells (T^a) or with T^a in the presence of MR1 anti-p39 antibody (*aP39*), or 2C11 anti-CD3 antibody (*aCD3*), at 10 μg/ml for 4 h, after which nuclear extracts were prepared. T cells were exposed to antibody for 15 min before B cells were added. Nuclear extracts were also obtained from B cells alone. (*B*) B cells were incubated with an equal number of T^a, with T^a in the presence of soluble CD40-Ig fusion protein (*CD40*) at 3.3 μg/ml, with LPS at 25 μg/ml, or with LPS in the presence of CD40-Ig. T cells were exposed to CD40-Ig for 15 min before B cells were added. Nuclear extracts were also obtained from B cells alone and from T^a alone. Nuclear extracts were tested by EMSA. The unmarked lanes represent κB probe alone. (◀) Position of specific κB-binding activity. In each case, one of two comparable experiments is shown.

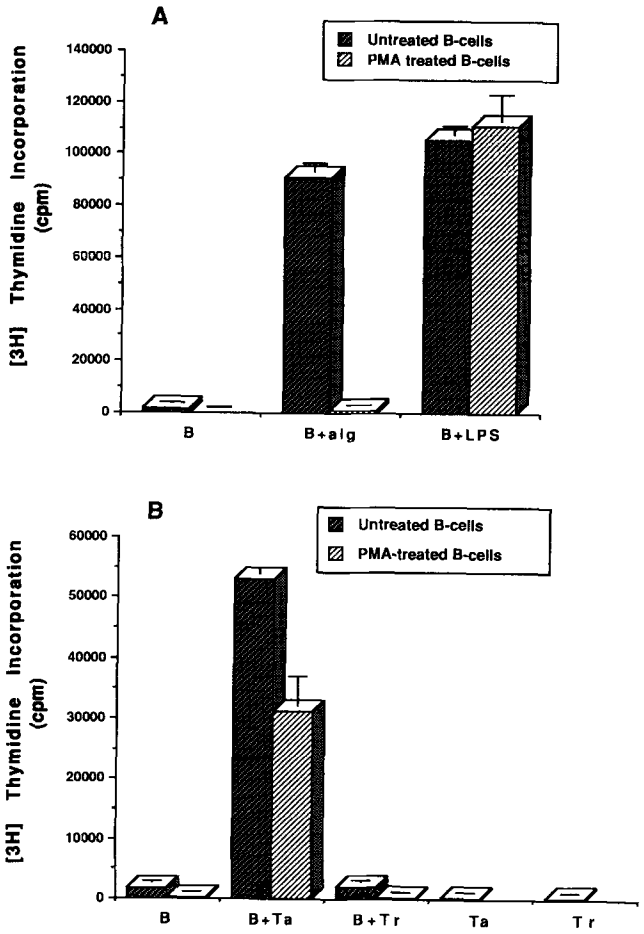

Figure 3. S phase responses of PKC-depleted B cells to fixed, activated T cells. Primary splenic B cells were treated with PMA at 100 ng/ml for 20 h to deplete PKC (*light crosshatch*), or incubated for the same length of time in medium alone (*dark crosshatch*). These two B cell populations were cultured separately with either (*A*) F(ab')$_2$GaMIgM at 10 μg/ml (*aIg*), lipopolysaccharide at 25 μg/ml (*LPS*), or medium alone (*B*); or (*B*) an equal number of fixed, activated T cells (T^a), an equal number of fixed, nonactivated or resting T cells (T^r), or medium alone (*B*), as indicated, for 48 h. In addition, T^a and T^r were incubated in medium alone. Incorporation of label was assessed after exposure of cells to [^{3}H]thymidine during the last 6 h of culture. Mean values of quadruplicate cultures are shown along with lines indicating the SE of the means. One of two comparable experiments is shown.

tion of NF-κB by LPS. These results strongly suggest that induction of B cell NF-κB by T^a depends on the interaction of p39 with its putative receptor, CD40, although the participation of other molecules has not been ruled out.

Lack of a Requirement for PKC in NF-κB Induction by T^a. Whereas B cell stimulation brought about by sIg ligation requires PKC (5, 12), this does not appear to be the case for B cell stimulation induced by T^a. As shown in Fig. 3, depletion of B cell PKC by treatment overnight with PMA somewhat reduced, but did not eliminate, [^{3}H]thymidine incorporation stimulated by T^a. In three experiments, stimulation of PMA-treated B cells by T^a amounted to 31–59% of the stimulation of untreated B cells. In contrast, PKC depletion completely eliminated the response of B cells to anti-Ig, as previously suggested (13). The response to LPS, which has been reported to be PKC independent (10), was not affected by PKC depletion. Thus, unlike (T independent) B cell stimulation mediated by sIg, B cell stimulation induced by activated T cells is not absolutely dependent on PKC (8).

To evaluate the signaling pathway required for T^a-induced NF-κB expression, B cells treated with PMA to deplete PKC and untreated B cells were stimulated in various ways, after which nuclear extracts were obtained and tested for the presence of NF-κB by EMSA. Results are shown in Fig. 4. PMA-treated and untreated B cells expressed similar levels of NF-κB at baseline. As expected on the basis of previous work from this laboratory, PKC depletion completely eliminated NF-κB induction produced by anti-Ig (as well as by the direct PKC agonist, PMA) but had little effect on induction of NF-κB by LPS (5). When B cells were stimulated by T^a, there was no reduction in NF-κB expression resulting from PKC depletion. In fact, the induction of NF-κB by T^a was enhanced in PMA-treated as opposed to untreated B cells. Thus, in direct contrast to B cell stimulation mediated by sIg, T cells induce NF-κB in B cells through a PKC-independent pathway.

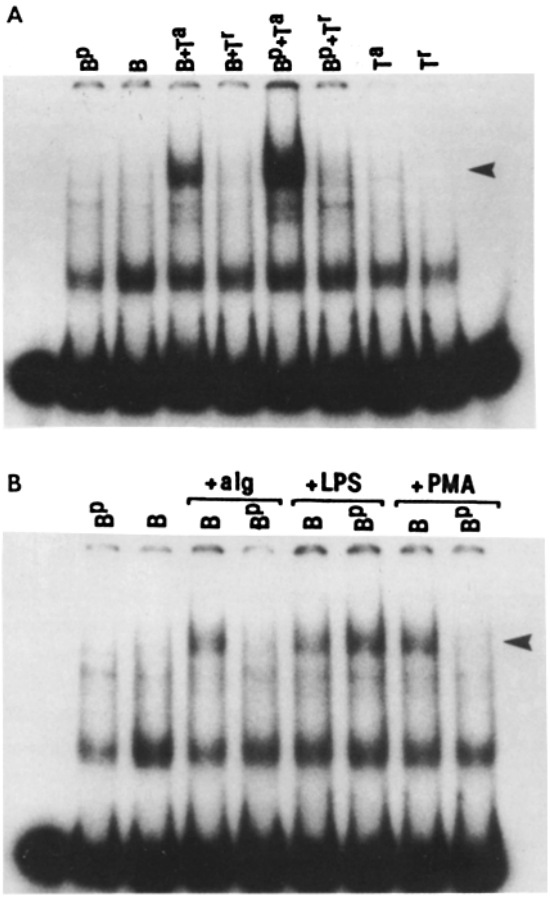

Figure 4. Induction of NF-κB in PKC-depleted B cells stimulated by fixed, activated T cells. Primary splenic B cells were treated with PMA at 100 ng/ml for 20 h to deplete PKC (B^p), or incubated for the same length of time in medium alone (B). These two B cell populations were cultured separately in the same experiment with (A) either an equal number of fixed, activated T cells (T^a), an equal number of fixed, nonactivated or resting T cells (T^r), or medium alone, or (B) either F(ab')$_2$GaMIgM at 10 μg/ml (*aIg*), lipopolysaccharide at 25 μg/ml (*LPS*), phorbol myristate acetate at 100 ng/ml (*PMA*), or medium alone, as indicated, for 2 h. In addition, T^a and T^r were incubated in medium alone (A). Nuclear extracts were prepared and tested by EMSA. The unmarked lanes represent κB probe alone. (◀) Position of specific κB-binding activity. One of two comparable experiments is shown.

Discussion

Utilizing a model for the efferent phase of T-B cell interaction, the present results show that T-dependent B cell stimulation is accompanied by early induction in B cells of the *trans*-acting transcription factor, NF-κB. This induction appears to be dependent on the availability of T cell p39 and B cell CD40 and is presumably triggered via the p39/CD40 ligand/receptor pair. The present work enlarges the set of surface receptors whose intracellular signaling results in nuclear expression of NF-κB, which presently includes sIg, TCR, and IL-2R, and the receptors for IL-1 and TNF-α (4, 5, 14–18).

In primary B cells, anti-Ig and T^a induce similar levels of nuclear NF-κB, although the time course for T^a induction is somewhat more prolonged than that for anti-Ig (5). However, these two modes of stimulating NF-κB completely diverge when the mechanism underlying NF-κB induction is assessed, in that anti-Ig–induced NF-κB requires PKC whereas T^a-induced NF-κB does not. By demonstrating that PKC does not play a primary role in T^a-mediated induction of NF-κB, the present results provide new insight into the intracellular signaling mechanism(s) triggered by T^a and indicate that T cells do not simply utilize upstream elements of the pathway that has been defined for sIg ligation. This accords well with the failure to demonstrate PKC activation in B cells stimulated by plasma membranes obtained from activated T cells recently observed by Dr. R. J. Noelle (personal communication). Although the observation that T cells stimulate cAMP in B cells (19) might suggest that NF-κB induction results from PKA-mediated phosphorylation of IκB (20), this target for PKA is not universally accepted (21).

Whereas PKC depletion did not impair induction of NF-κB by T^a at all, S phase entry was reduced somewhat. Notwithstanding the fact that both responses still proceeded in the absence of PKC, this difference emphasizes that no single transcription factor is likely to play an overarching role in determining S phase entry.

Although the discrete mechanism responsible for T-dependent induction of NF-κB remains to be elucidated, the present work clearly demonstrates that T cells (presumably via B cell CD40) and sIg utilizes different intracellular signaling pathways to stimulate nuclear NF-κB expression. Thus, in the same cell type two separate receptors appear to induce the same transcription factor yet do so via separate mechanisms. This suggests the possibility that NF-κB may be one of an unknown number of transcription factors that take part in a final common pathway for B cell stimulation, responsive to divergent receptor/signaling pathways, but always involved in regulating the nuclear transcriptional events that are associated with growth control.

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